


VERIFICATION OF TRANSLATION

Re: JAPANESE PATENT APPLICATION NO. 2000-23581

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hereby declare that I am the translator of the  
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true translation to the best of my knowledge and  
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[Document Name] Specification

[Title of the Invention] Method for Determining HIV-1  
Subtype

[Claims]

5           [Claim 1] A method for determining HIV-1 subtypes,  
which comprises the steps of amplifying nucleic acid using  
as a target sequence a portion of a nucleotide sequence of  
the env gene of HIV-1 wherein at least one of the 5'  
terminal and 3' terminal nucleotide sequences is different  
10 depending on the HIV-1 subtype, and detecting the subtype  
depending on whether or not the nucleic acid has been  
amplified.

          [Claim 2] The method according to Claim 1, wherein  
the target sequence is 100 to 2500 nucleotides long.

15           [Claim 3] The method according to Claim 1 or Claim 2,  
wherein the sequence from the 1st through 30th bases from  
the 3' terminal and/or 5' terminal of the target sequence  
is different depending on the subtype.

          [Claim 4] The method according to Claim 3, wherein  
20 the 3' terminal of the target sequence is in the C3 region  
of the env gene of HIV-1.

          [Claim 5] The method according to Claim 4, wherein  
the 5' terminal of the target sequence is in the C2 region  
of the env gene of HIV-1.

25           [Claim 6] The method according to any of Claims 1

through 5, wherein different amplification reactions are carried out using different primer pairs, to detect different subtypes.

[Claim 7] The method according to Claim 6, wherein  
5 at least two different subtypes are detected by carrying out amplification reactions at least twice with different primer pairs consisting of a primer (primer 1) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 1) that differs  
10 depending on subtype in the C3 region of the env gene of HIV-1, and a primer (primer 2) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 2) of the C2 region of the env gene of HIV-1.

15 [Claim 8] The method according to any of Claims 1 through 7, wherein the first amplification reaction is carried out with the first primer pair using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1, and then the second amplification reaction  
20 is carried out with the second primer pair using as a target sequence a portion of said target sequence wherein at least one of the 5' terminal and 3' terminal nucleotide sequences is different depending on the subtype, to detect the subtype depending on whether or not the nucleic acid  
25 has been amplified by the second amplification reaction.

[Claim 9] The method according to Claim 8, wherein the second primer pair consists of a primer (primer 1) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 1) that differs  
5 depending on subtype in the C3 region of the env gene of HIV-1, and a primer (primer 2) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 2) of the C2 region of the env gene of HIV-1; and the first primer pair consists of a primer  
10 (primer 3) that includes a sequence complementary to a portion of a nucleotide sequence (nucleotide sequence 3) of the region downstream of the 3' terminal of nucleotide sequence 1 of the env gene of HIV-1, and a primer (primer 4) that includes a sequence complementary to a portion of  
15 a nucleotide sequence (nucleotide sequence 4) of the region upstream of the 5' terminal of nucleotide sequence 2 of the env gene of HIV-1.

[Claim 10] The method according to Claim 8 or Claim 9, wherein at least two subtypes are distinguished by  
20 repeating at least once, with different second primer pairs, a series of operations comprising: the first amplification reaction that is carried out with the first primer pair using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1; the second  
25 amplification reaction that is then carried out with the

second primer pair using as a target sequence a nucleotide sequence within said target sequence; to detect subtypes depending on whether or not the nucleic acid has been amplified by the second amplification reaction.

5           [Claim 11] The method according to Claim 10, wherein subtypes A, B, C, and E are distinguished by:

          (a) detecting subtype A using as the first primer pair a mixture of primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and  
10   primer 12B containing nucleotide sequence ACAGTAGAAAAATTCTCCTC (Sequence ID No. 6), and a mixture of primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG  
15   (Sequence ID No. 9), and using as the second primer pair, a mixture of primer 11QA1 containing nucleotide sequence CTCCTGAGGAGTTAGCAAAG (Sequence ID No. 27) and primer 10U containing nucleotide sequence CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20);

20           (b) detecting subtype B using as the first primer pair a mixture of primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 12B containing nucleotide sequence ACAGTAGAAAAATTCTCCTC (Sequence ID No. 6), and a mixture of  
25   primer 9AE containing nucleotide sequence

CACAGTACAATGCACACATG (Sequence ID No. 8) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the second primer pair a mixture of primer 11VB containing nucleotide sequence  
5 CACAATTAAACTGTGCATTAC (Sequence ID No. 28) and primer 10U containing nucleotide sequence CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20);

(c) detecting subtype C using as the first primer pair a mixture of primer 12A containing nucleotide  
10 sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 12B containing nucleotide sequence ACAGTAGAAAAATTCTCCTC (Sequence ID No. 6), and a mixture of primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8) and primer 9B  
15 containing nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the second primer pair a mixture of primer 11XC containing nucleotide sequence TTGTTTTATTAGGGAAGTGTTT (Sequence ID No. 29) and primer 10UC containing nucleotide sequence CTGTTAAATGGTAGTCTAGC  
20 (Sequence ID No. 24); and

(d) detecting subtype E using as the first primer pair a mixture of primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 12B containing nucleotide sequence  
25 ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6), and a mixture of

primer 9AE containing nucleotide sequence  
CACAGTACAATGCACACATG (Sequence ID No. 8) and primer 9B  
containing nucleotide sequence CACAGTACAATGTACACATG  
(Sequence ID No. 9), and using as the second primer pair a  
5 mixture of primer 11WE containing nucleotide sequence  
CTCTACAATTAAAATGATGCATTG (Sequence ID No. 30) and primer  
10U containing nucleotide sequence CTGTTAAATGGCAGTCTAGC  
(Sequence ID No. 20).

[Claim 12] The method according to Claim 8 or Claim  
10 9, wherein at least two subtypes are distinguished by  
repeating at least once, with different first and second  
primer pairs, a series of operations comprising: the first  
amplification reaction that is carried out with the first  
primer pair using as a target sequence a portion of a  
15 nucleotide sequence of the env gene of HIV-1; the second  
amplification reaction that is then carried out with the  
second primer pair using as a target sequence a nucleotide  
sequence within said target sequence; to detect subtypes  
depending on whether or not the nucleic acid has been  
20 amplified by the second amplification reaction.

[Claim 13] The method according to Claim 12, wherein  
subtypes A, B, and E are distinguished by:

(a) detecting subtype A using as the first primer  
pair primer 12A containing nucleotide sequence  
25 GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 9AE

containing nucleotide sequence CACAGTACAATGCACACATG  
(Sequence ID No. 8), and using as the second primer pair a  
mixture of primer 11QA containing nucleotide sequence  
CTCCTGAGGGGTTAGCAAAG (Sequence ID No. 1) and primer 10  
5 containing nucleotide sequence AAATGGCAGTCTAGCAGAAG  
(Sequence ID No. 4);

(b) detecting subtype B using as the first primer  
pair primer 12B containing nucleotide sequence  
ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6) and primer 9B  
10 containing nucleotide sequence CACAGTACAATGTACACATG  
(Sequence ID No. 9), and using as the second primer pair a  
mixture of primer 11BB containing nucleotide sequence  
CTGTGCATTACAATTTCTGG (Sequence ID No. 2) and primer 10  
containing nucleotide sequence AAATGGCAGTCTAGCAGAAG  
15 (Sequence ID No. 4); and

(c) detecting subtype E using as the first primer  
pair primer 12E containing nucleotide sequence  
GCAATAGAAAAATTCCCCTC (Sequence ID No. 7) and primer 9AE  
containing nucleotide sequence CACAGTACAATGCACACATG  
20 (Sequence ID No. 8), and using as the second primer pair a  
mixture of primer 11QE containing nucleotide sequence  
CTCCTGAGGGTGGTTGAAAG (Sequence ID No. 3) and primer 10  
containing nucleotide sequence AAATGGCAGTCTAGCAGAAG  
(Sequence ID No. 4).

25 [Claim 14] The method according to any of Claims 1



through 13, which further comprises the steps of  
amplifying nucleic acid using as a target sequence a  
portion of a nucleotide sequence of the HIV-1 genome, the  
nucleotide sequence being highly conserved among all  
5 subtypes, and ascertaining the presence or absence of HIV-  
1 depending on whether or not the nucleic acid has been  
amplified.

[Claim 15] The method according to Claim 14, wherein  
the step for ascertaining the presence or absence of HIV-1  
10 comprises amplifying the nucleic acid with the first  
primer pair using as a target sequence a portion of a  
nucleotide sequence of the HIV-1 genome, the nucleotide  
sequence being highly conserved among all subtypes, then  
carrying out the second amplification reaction with the  
15 second primer pair using as a target sequence a nucleotide  
sequence within the above target sequence, and  
ascertaining the presence or absence of HIV-1 depending on  
whether or not the nucleic acid has been amplified.

[Claim 16] The method according to Claim 15, wherein  
20 the primers that are used comprise a mixture of a  
plurality of upstream primers with different nucleotide  
sequences and a plurality of downstream primers with  
different nucleotide sequences.

[Claim 17] The method according to Claim 16, wherein  
25 the first primer pair comprises a mixture of primer 12A

containing nucleotide sequence GCAATAGAAAAATTCTCCTC  
(Sequence ID No. 5), primer 12B containing nucleotide  
sequence ACAGTAGAAAAATTCTCCTC (Sequence ID No. 6), primer  
9AE containing nucleotide sequence CACAGTACAATGCACACATG  
5 (Sequence ID No. 8), and primer 9B nucleotide sequence  
CACAGTACAATGTACACATG (Sequence ID No. 9), and the second  
primer pair comprises primer 11LB containing nucleotide  
sequence AATTTCTGGGTCCCCTCCTG (Sequence ID No. 18), primer  
11LAE containing nucleotide sequence AATTTCTAGATCCCCTCCTG  
10 (Sequence ID No. 25), primer 11LC containing nucleotide  
sequence AATTTCTAGGTCCCCTCCTG (Sequence ID No. 26), and  
primer 10U containing nucleotide sequence  
CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20).

[Claim 18] A kit for determining HIV-1 subtypes,  
15 comprising a primer pair in which a target sequence is a  
portion of a nucleotide sequence of the env gene of HIV-1  
wherein at least one of the 5' terminal and 3' terminal  
nucleotide sequences is different depending on the subtype.

20 [Detailed Description of the Invention]

[0001]

[Technical Field to Which the Invention Pertains]

The present invention relates to a method for  
determining HIV-1 subtypes, and a kit for determining HIV-  
25 1 subtypes.

[0002]

[Prior Art]

The human immunodeficiency virus (hereinafter, referred to as "HIV") is a virus causing acquired immune deficiency syndrome (hereinafter, referred to as "AIDS"),  
5 and type 1 (HIV-1) and type 2 (HIV-2) are known. Most cases involve HIV-1, for which various subtypes have been discovered.

Determining the HIV-1 subtype in infected  
10 individuals is important for assessing the reliability of virological test results (particularly the drug resistance based on genotype or the determination of plasma HIV-1 RNA concentration) and the route of infection. HIV-1 subtypes are generally determined through the sequencing of  
15 specific regions of the virus genome and phylogenetic analysis of the results, but these are complicated and expensive procedures.

[0003]

[Problem to be Solved by the Invention]

20 Thus, an object of the present invention is to provide a simpler method for determining HIV-1 subtypes.

Another object of the present invention is to provide a kit for determining HIV-1 subtypes.

[0004]

25 [Means for Solving the Problem]

The inventors have designed various subtype-specific primers and have successfully used them to amplify nucleic acid in samples for rapid determination of HIV-1 subtypes thereby to complete the present invention.

5           Specifically, the present invention provides a method for determining HIV-1 subtypes, which comprises the steps of amplifying nucleic acid using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1 wherein at least one of the 5' terminal and  
10   3' terminal nucleotide sequences is different depending on the HIV-1 subtype, and detecting the subtype depending on whether or not the nucleic acid has been amplified. The target sequence should be 100 to 2500 base pairs in length, and preferably 150 to 500 base pairs in length. In the  
15   above method, the sequence from the 1st through 30th bases from the 3' terminal and/or 5' terminal of the target sequence should be different depending on the subtype. For example, the 3' terminal of the target sequence may be in the C3 region of the env gene of HIV-1. The 5'  
20   terminal of the target sequence may be in the C2 region of the env gene of HIV-1. Different subtypes can be detected by different amplification reactions using different primer pairs. For example, at least two subtypes can be detected by carrying out amplification reactions at least  
25   twice with different primer pairs consisting of a primer

(primer 1) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 1) that differs depending on subtype in the C3 region of the env gene of HIV-1, and a primer (primer 2) that includes a  
5 sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 2) of the C2 region of the env gene of HIV-1.

[0005]

The first amplification reaction may be carried out  
10 with the first primer pair using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1, the second amplification reaction may then be carried out with the second primer pair using as a target sequence a portion of the aforementioned target sequence, wherein at  
15 least one of the 5' terminal and 3' terminal nucleotide sequences is different depending on the HIV-1 subtype, and the subtype may be detected depending on whether or not the nucleic acid has been amplified by the second amplification reaction. For example, the second primer  
20 pair consist of a primer (primer 1) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 1) that differs depending on subtype in the C3 region of the env gene of HIV-1, and a primer (primer 2) that includes a sequence complementary  
25 to a portion of the nucleotide sequence (nucleotide

sequence 2) in the C2 region of the env gene of HIV-1; and  
the first primer pair may consist of a primer (primer 3)  
that includes a sequence complementary to a portion of a  
nucleotide sequence (nucleotide sequence 3) of a region  
5 downstream of the 3' terminal of nucleotide sequence 1 of  
the env gene of HIV-1, and a primer (primer 4) that  
includes a sequence complementary to a portion of the  
nucleotide sequence (nucleotide sequence 4) of a region  
upstream of the 5' terminal of nucleotide sequence 2 of  
10 the env gene of HIV-1.

[0006]

At least two subtypes can be distinguished by  
repeating at least once the following series of operations  
with different second primer pairs, the operations  
15 comprising the first amplification reaction that is  
carried out with the first primer pair using as a target  
sequence a portion of a nucleotide sequence of the env  
gene of HIV-1, the second amplification reaction that is  
then carried out with the second primer pair using as a  
20 target sequence a nucleotide sequence within the above  
target sequence, and the detection of subtypes depending  
on whether or not the nucleic acid has been amplified by  
the second amplification reaction. For example, subtypes  
A, B, C, and E can be distinguished by: (a) detecting  
25 subtype A using as the first primer a mixture of primer

12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC  
(Sequence ID No. 5), primer 12B containing nucleotide  
sequence ACAGTAGAAAAATTCTCCTC (Sequence ID No. 6), primer  
9AE containing nucleotide sequence CACAGTACAATGCACACATG  
5 (Sequence ID No. 8) and primer 9B containing nucleotide  
sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and  
using as the second primer pair a mixture of primer 11QA1  
containing nucleotide sequence CTCCTGAGGAGTTAGCAAAG  
(Sequence ID No. 27) and primer 10U containing nucleotide  
10 sequence CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20);

(b) detecting subtype B using as the first primer a  
mixture of primer 12A containing nucleotide sequence  
GCAATAGAAAAATTCTCCTC (Sequence ID No. 5), primer 12B  
containing nucleotide sequence ACAGTAGAAAAATTCTCCTC  
15 (Sequence ID No. 6), primer 9AE containing nucleotide  
sequence CACAGTACAATGCACACATG (Sequence ID No. 8) and  
primer 9B containing nucleotide sequence  
CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the  
second primer pair a mixture of primer 11VB containing  
20 nucleotide sequence CACAATTAAACTGTGCATTAC (Sequence ID  
No. 28) and primer 10U containing nucleotide sequence  
CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20);

(c) detecting subtype C using as the first primer a  
mixture of primer 12A containing nucleotide sequence  
25 GCAATAGAAAAATTCTCCTC (Sequence ID No. 5), primer 12B

containing nucleotide sequence ACAGTAGAAAAATTCTCCTC  
(Sequence ID No. 6), primer 9AE containing nucleotide  
sequence CACAGTACAATGCACACATG (Sequence ID No. 8) and  
primer 9B containing nucleotide sequence  
5 CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the  
second primer pair a mixture of primer 11XC containing  
nucleotide sequence TTGTTTTATTAGGGAAGTGTTTC (Sequence ID  
No. 29) and primer 10U containing nucleotide sequence  
CTGTAAATGGCAGTCTAGC (Sequence ID No. 24); and  
10 (d) detecting subtype C using as the first primer a  
mixture of primer 12A containing nucleotide sequence  
GCAATAGAAAAATTCTCCTC (Sequence ID No. 5), primer 12B  
containing nucleotide sequence ACAGTAGAAAAATTCTCCTC  
(Sequence ID No. 6), primer 9AE containing nucleotide  
15 sequence CACAGTACAATGCACACATG (Sequence ID No. 8) and  
primer 9B containing nucleotide sequence  
CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the  
second primer pair a mixture of primer 11WE containing  
nucleotide sequence CTCTACAATTAAAATGATGCATTG (Sequence ID  
20 No. 30) and primer 10U containing nucleotide sequence  
CTGTAAATGGCAGTCTAGC (Sequence ID No. 20).

[0007]

Alternatively, at least two subtypes can be  
distinguished by repeating at least once the following  
25 series of operations with different first and second



primer pairs, the operations comprising the first amplification reaction that is carried out with the first primer pair using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1, the second  
5 amplification reaction that is then carried out with the second primer pair using as a target sequence a nucleotide sequence within the target sequence in the first reaction, and the detection of subtypes depending on whether or not the nucleic acid has been amplified by the second  
10 amplification reaction. For example, subtypes A, B, and E can be distinguished by: (a) detecting subtype A using as the first primer pair primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 9AE containing nucleotide sequence  
15 CACAGTACAATGCACACATG (Sequence ID No. 8), and using as the second primer pair primer 11QA containing nucleotide sequence CTCCTGAGGGGTTAGCAAAG (Sequence ID No. 1) and primer 10 containing nucleotide sequence  
AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4);  
20 (b) detecting subtype B using as the first primer pair primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the second primer pair a  
25 mixture of primer 11BB containing nucleotide sequence

CTGTGCATTACAATTTCTGG (Sequence ID No. 2) and primer 10  
containing nucleotide sequence AAATGGCAGTCTAGCAGAAG  
(Sequence ID No. 4); and

(c) detecting subtype E using as the first primer  
5 pair primer 12E containing nucleotide sequence  
GCAATAGAAAAATTCCCCTC (Sequence ID No. 7) and primer 9AE  
containing nucleotide sequence CACAGTACAATGCACACATG  
(Sequence ID No. 8), and using as the second primer pair a  
mixture of primer 11QE containing nucleotide sequence  
10 CTCCTGAGGGTGGTTGAAAG (Sequence ID No. 3) and primer 10  
containing nucleotide sequence AAATGGCAGTCTAGCAGAAG  
(Sequence ID No. 4).

[0008]

The method of the present invention may further  
15 comprise the steps of amplifying nucleic acid using as a  
target sequence a portion of a nucleotide sequence of the  
HIV-1 genome, the nucleotide sequence being highly  
conserved among all subtypes and ascertaining the presence  
or absence of HIV-1 depending on whether or not the  
20 nucleic acid has been amplified. The step for  
ascertaining the presence or absence of HIV-1 comprises  
amplifying the nucleic acid with the first primer pair  
using as a target sequence a portion of a nucleotide  
sequence of the HIV-1 genome, the nucleotide sequence  
25 being highly conserved among all subtypes, then carrying

out the second amplification reaction with the second primer pair using as a target sequence a nucleotide sequence within the above target sequence, and ascertaining the presence or absence of HIV-1 depending on whether or not the nucleic acid has been amplified. The first primers referred to here may comprise a mixture of primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5), primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6), primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8), and primer 9B nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and the second primers may comprise a mixture of primer 11LB containing nucleotide sequence AATTTCTGGGTCCCCTCCTG (Sequence ID No. 18), primer 11LAE containing nucleotide sequence AATTTCTAGATCCCCTCCTG (Sequence ID No. 25), primer 11LC containing nucleotide sequence AATTTCTAGGTCCCCTCCTG (Sequence ID No. 26), and primer 10U containing nucleotide sequence CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20).

[0009]

Another object of the present invention is to provide a kit for determining HIV-1 subtypes, comprising a primer pair in which a target sequence is a portion of a nucleotide sequence of the env gene of HIV-1 wherein at

least one of the 5' terminal and 3' terminal nucleotide sequences is different depending on the subtype.

[0010]

[Mode for Carrying out the Invention]

5           Hereinafter, embodiments of the present invention are illustrated below.

          A sample of blood, lymph, spinal fluid, semen, lymph node, or the like is taken from individuals suspected of HIV-1 infection, infected individuals and patients  
10 confirmed with HIV-1 infection, patients being treated for HIV-1, and the like. DNA is extracted using a QIAamp Blood Kit (QIAGEN), either directly or after monocytes have been isolated from the sample Ficoll-Paque density gradient centrifugation (Pharmacia). Alternatively, RNA  
15 is extracted using a QIAamp Viral RNA Kit by QIAGEN from plasma. The DNA or RNA concentration is then determined based on the absorption at the wavelength of 260 nm.

[0011]

          The nucleic acid is then treated in PCR, and  
20 preferably nested PCR.

          The use of nested PCR is described below. Nested PCR involves designing the second primer pair inside a target sequence amplified with another primer pair (first primer pair), carrying out the first PCR step, and then  
25 diluting the reaction product as a new template for the

second PCR step, and then carrying out the second PCR step. Undesirable sequences are sometimes amplified in addition to the target sequence in the first PCR step. However, there is very little possibility that undesirable

5 fragments amplified during the first PCR step have a sequence with which the primers of the second primer pair will anneal. The second PCR step is thus carried out for selectively amplifying the target sequence.

[0012]

10 The initial PCR step (first PCR) is first carried out using different primer pairs specific to each subtype to be distinguished (such as subtype A, subtype B, and subtype E). Alternatively, universal primer pairs allowing any type of subtypes to be amplified can be used  
15 instead of subtype-specific primer pairs.

[0013]

An example of a subtype-specific primer pair is a primer pair consisting of a primer (primer 4') which includes a sequence complementary to a portion of a  
20 nucleotide sequence in the C2 region of the env gene of HIV-1 and a primer (primer 3') which includes a sequence complementary to a portion of the nucleotide sequence of the C3 region of the env gene for HIV-1 that differs depending on subtype (that is, subtype-specific nucleotide  
25 sequence). Since the C2 region of the env gene of HIV-1

has a nucleotide sequence that differs depending on the subtype, as shown in Figure 1, the nucleotide sequence may be selected from this region to design primer 4'. Because the C3 region of the env gene of HIV-1 varies depending on the subtype, as shown in Figure 2, the nucleotide sequence may be selected from this region to design primer 3'. The primer should generally be 18 to 30 base pairs, and preferably 20 to 25 base pairs in length. Specifically, the following primers can be used.

10 [0014]

A primer pair for the first PCR to detect subtype A and their nucleotide sequences

9AE/12A

primer 9AE: CACAGTACAATGCACACATG (Sequence ID No. 8)

15 primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

Primer 9AE is a subtype A, E, F, and H-specific primer in which the sequence is complementary to the sequence from 6943 to 6962, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

20 [0015]

Primer 12A is a subtype A, C, E, G, H, I, and J-specific primer in which the sequence is complementary to the sequence from 7369 to 7350, counting from the 5' terminal (left terminal), of the complete base sequence

25

for HIV-1 (NL4-3 strain).

[0016]

A primer pair for the first PCR to detect subtype B and their nucleotide sequences

5 9B/12B

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12B: ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6)

Primer 9B is a subtype B, C, D, E, F, G, H, and J-specific primer in which the sequence is complementary to  
10 the sequence from 6943 to 6962, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 12B is a subtype B, D, E, F, and I-specific primer in which the sequence is complementary to the  
15 sequence from 7369 to 7350, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0017]

A primer pair for the first PCR to detect subtype E  
20 and their nucleotide sequences

9AE/12E

primer 9AE: CACAGTACAATGCACACATG (Sequence ID No. 8)

primer 12E: GCAATAGAAAAATTCCCCTC (Sequence ID No. 7)

Primer 12E is a primer specific to subtype E only,  
25 in which the sequence is complementary to the sequence

from 6943 to 6962, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0018]

- 5           A primer pair for first PCR to detect subtype C and their nucleotide sequences

9B/12A

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

- 10          A primer pair for the first PCR to detect subtype D and their nucleotide sequences

9B/12B

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12B: ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6)

- 15          A primer pair for the first PCR to detect subtype F and their nucleotide sequences

9B/12A

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

- 20          A primer pair for the first PCR to detect subtype G and their nucleotide sequences

9B/12A

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

- 25          Primer pairs for first PCR to detect subtype H and



their nucleotide sequences

9B/12A

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

5       The first PCR may alternatively be carried out using  
a primer mixture capable of giving amplified products for  
several subtypes. An example of a primer for such a  
purpose is a mixture of primers 9AE, primer 9B, primer 12A,  
and primer 12B.

10       [0019]

1/1000 to 1/5 (for example, 1/50) of the PCR  
products is used to carry out the next PCR (second PCR)  
with another primer pair specific to each subtype. The  
primer pair for the second PCR is designed from within the  
15 target sequence amplified during the first PCR. For  
example, at least one of the primers forming the subtype-  
specific primer pair for second PCR can be a primer  
(primer 1) containing a sequence complementary to a  
portion of the subtype-specific nucleotide sequence of the  
20 C2 region of the env gene for HIV-1. Since the nucleotide  
sequence of the C2 region of the env gene for HIV-1  
differs depending on subtype, as shown in Figure 1, a  
nucleotide sequence from this region can be selected to  
design the primer. Figure 2 gives the nucleotide sequence  
25 of the 3' adjacent region (C3 region) of the V3 region of

the env gene for various subtypes of HIV-1. Since the nucleotide sequence varies depending on the subtype, a suitable sequence can be selected to design a primer. To design a subtype-specific primer, phylogenetic analysis is employed to select nucleotide sequences of a given subtype which are as genetically remote as possible from the corresponding nucleotide sequences of other subtypes. An example can include a primer (primer 2) containing a sequence complementary to a portion of the nucleotide sequence of the C3 region of the env gene for HIV-1. Specifically, the primer pairs containing the following nucleotide sequences can be used.

[0020]

A primer pair for the second PCR to detect subtype A and their nucleotide sequences

10/11QA

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11QA: CTCCTGAGGGGTTAGCAAAG (Sequence ID No. 1)

Primer 10 is a subtype A, B, D, and E-specific primer in which the sequence is complementary to the sequence from 6997 to 7016, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11QA is a primer specific to only subtype A,

in which the sequence is complementary to the sequence from 7313 to 7294, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

5 [0021]

10U/11QA1

primer 10U: CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20)

primer 11QA1: CTCCTGAGGAGTTAGCAAAG (Sequence ID No. 27)

Primer 10U is a subtype A, B, D, E, and J-specific  
10 primer in which the sequence is complementary to the sequence from 6992 to 7011, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11QA1 is a primer specific to subtype A only,  
15 in which the sequence is complementary to the sequence from 7313 to 7294, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0022]

20 A primer pair for the second PCR to detect subtype B and their nucleotide sequences

10/11BB

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11BB: CTGTGCATTACAATTTCTGG (Sequence ID No. 2)

25 Primer 11BB is a primer specific to only subtype B,

in which the sequence is complementary to the sequence from 7338 to 7319, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

5           10U/11VB

primer 10U: CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20)

primer 11VB: CACAATTAAACTGTGCATTAC (Sequence ID No. 28)

Primer 11VB is a primer specific to only subtype B, in which the sequence is complementary to the sequence from 7349 to 7328, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0023]

A primer pair for the second PCR to detect subtype E and their nucleotide sequences

15           10/11QE

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11QE: CTCCTGAGGGTGGTTGAAAG (Sequence ID No. 3)

Primer 11QE is a primer specific to only subtype E, in which the sequence is complementary to the sequence from 7313 to 7294, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

10U/11WE

25   primer 10U: CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20)

primer 11WE: CTCTACAATTAAAATGATGCATTG (Sequence ID No. 30)

Primer 11WE is a primer specific to only subtype E, in which the sequence is complementary to the sequence from 7352 to 7339, counting from the 5' terminal (left  
5 terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0024]

A primer pair for the second PCR to detect subtype C and their nucleotide sequences

10 10C/11RC

primer 10C: AAATGGTAGCCTAGCAGAAG (Sequence ID No. 10)

primer 11RC: CTCCTGAGGATGGTGCAAATTT (Sequence ID No. 13)

Primer 10C is a subtype C and F-specific primer in which the sequence is complementary to the sequence from  
15 6997 to 7016, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0025]

Primer 11RC is a primer specific to only subtype C,  
20 in which the sequence is complementary to the sequence from 7313 to 7292, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

10U/11XC

25 primer 10U: CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20)

primer 11XC: TTGTTTTATTAGGGAAGTGTTTC (Sequence ID No. 29)

Primer 11XC is a primer specific to only subtype C, in which the sequence is complementary to the sequence from 7289 to 7268, counting from the 5' terminal (left  
5 terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0026]

A primer pair for the second PCR to detect subtype D and their nucleotide sequences

10 10/11RD

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11RD: CTCCTGAGGATGGTTTAAAAAT (Sequence ID No. 14)

Primer 11RD is a primer specific to only subtype D, in which the sequence is complementary to the sequence  
15 from 7313 to 7292, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0027]

A primer pair for the second PCR to detect subtype F  
20 and their nucleotide sequences

10C/11RF

primer 10C: AAATGGTAGCCTAGCAGAAG (Sequence ID No. 10)

primer 11RF: CTCCTGAGGATGAGTTAAATTT (Sequence ID No. 15)

Primer 11RF is a primer specific to only subtype F,  
25 in which the sequence is complementary to the sequence

from 7313 to 7292, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0028]

5           A primer pair for the second PCR to detect subtype G and their nucleotide sequences

10G/11SG

primer 10G: GAATGGCAGTTTAGCAGAAG (Sequence ID No. 11)

primer 11SG: TCCTGCAGATGAGTTAAAGG (Sequence ID No. 16)

10           Primer 10G is a primer specific to only subtype G, in which the sequence is complementary to the sequence from 6997 to 7016, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

15           Primer 11SG is a primer specific to only subtype G, in which the sequence is complementary to the sequence from 7312 to 7293, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

20           [0029]

          A primer pair for the second PCR to detect subtype H and their nucleotide sequences

10H/11SH

primer 10H: GTCAAATGGCAGTTTAGCAG (Sequence ID No. 12)

25   primer 11SH: TCCTGAGGATGGTTTAAAGG (Sequence ID No. 17)

Primer 10H is a primer specific to only subtype H, in which the sequence is complementary to the sequence from 6994 to 7013, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11SH is a primer specific to only subtype H, in which the sequence is complementary to the sequence from 7312 to 7293, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0030]

Second PCR may alternatively be carried out using a mixture of primers capable of giving amplified products for several subtypes in order to permit the amplification of any subtype. Examples of primers for that purpose include a mixture the following primer.

primer 10U: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)  
primer 11LB: AATTTCTGGGTCCCCTCCTG (Sequence ID No. 18)  
primer 11LAE: AATTTCTAGATCCCCTCCTG (Sequence ID No. 25)  
primer 11LC: AATTTCTAGGTCCCCTCCTG (Sequence ID No. 26)

[0031]

Primer 11LB is a subtype B, D, F, G, and I-specific primer in which the sequence is complementary to the sequence from 7327 to 7308, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1



(NL4-3 strain).

Primer 11LAE is a subtype A, E, F, G, I, and J-specific primer in which the sequence is complementary to the sequence from 7327 to 7308, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11LC is a subtype C, F, G, H, I, and J-specific primer in which the sequence is complementary to the sequence from 7327 to 7308, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0032]

The following primers can also be used.

Primer 10KC: CTCAACTACTGTTAAATGGTAG (Sequence ID No. 21)

Primer 10KC is a primer specific to only subtype C, in which the sequence is complementary to the sequence from 6984 to 7005, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0033]

Primer 10UF: CTGTTAAATGGCAGCCTAGC (Sequence ID No. 22)

Primer 10UF is a subtype A, E, F, H, and I-specific primer in which the sequence is complementary to the sequence from 6992 to 7011, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1

(NL4-3 strain).

Primer 10UG: CTGTTAAATGGCAGTTTAGC (Sequence ID No. 23)

Primer 10UG is a subtype A, E, G, I, and J-specific primer in which the sequence is complementary to the  
5 sequence from 6992 to 7011, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 10UC: CTGTTAAATGGTAGTCTAGC (Sequence ID No. 24)

Primer 10UC is a subtype C and E-specific primer in  
10 which the sequence is complementary to the sequence from 6992 to 7011, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0034]

15 Primer 11LE: AATTTCTAGATCTCCTCCTG (Sequence ID No. 19)

Primer 11LE is a subtype E, F, G, H, and J-specific primer in which the sequence is complementary to the  
sequence from 7327 to 7308, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1  
20 (NL4-3 strain).

Primer 11LC: AATTTCTAGGTCCCCTCCTG (Sequence ID No. 26)

Primer 11LC is a subtype C, F, G, H, I, and J-specific primer in which the sequence is complementary to  
the sequence from 7327 to 7308, counting from the 5'  
25 terminal (left terminal), of the complete base sequence

for HIV-1 (NL4-3 strain).

Primer 11TC: TTCTCCTCTACAATTAAAGC (Sequence ID No. 31)

Primer 11TC is a primer specific to only subtype C, in which the sequence is complementary to the sequence  
5 from 7357 to 7238, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

[0035]

Primer 11RC1: TTATTGTTTTATTAGGGAAGTG (Sequence ID No. 32)

10 Primer 11RC1 is a primer specific to only subtype C, in which the sequence is complementary to the sequence from 7292 to 7271, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

15 Primer 11SE: TGCATTGTAATTTCTAGATCTC (Sequence ID No. 33)

Primer 11SE is a primer specific to only subtype E, in which the sequence is complementary to the sequence from 7333 to 7314, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3  
20 strain).

Primer 11BE: TGATGCATTGTAATTTCTAG (Sequence ID No. 34)

Primer 11BE is a primer specific to only subtype E, in which the sequence is complementary to the sequence from 7338 to 7319, counting from the 5' terminal (left  
25 terminal), of the complete base sequence for HIV-1 (NL4-3

strain).

[0036]

The PCR procedures and reaction conditions may be in accordance with those in Bruisten S. et al., *AIDS Res Hum*  
5 *Retroviruses* 1993, 9:259-265, but the hot start method is preferred. In hot start PCR, the PCR reaction solution is kept on a hot plate for start up at an elevated temperature (usually 90°C or higher).

[0037]

10 However, it sometimes happens that no subtype is detected in an attempt to determine the HIV-1 subtype in such a method. Possible causes may be that the HIV-1 DNA concentration is below the detection threshold, or the presence of numerous variants at the primer binding site.

15 To deal with the former possibility, the above method can be implemented after extracting RNA from plasma and converting the RNA into cDNA using reverse transcriptase, since the concentration of HIV-1 is generally higher in plasma than in cells.

20 [0038]

To deal with the latter possibility, the determination of the subtype by the method of the present invention is held off, another genetic region of HIV-1 is amplified by PCR to determine the nucleotide sequence, and  
25 the subtype is determined by a conventional method (Note:

HIV-1 infection is generally diagnosed by detecting antibodies. This invention is not a method for diagnosing HIV-1 infection.).

The PCR reaction products are separated by agarose  
5 gel electrophoresis and detected by ethidium bromide  
staining. Although distinct bands can be observed with  
the use of primers consistent with the subtype of the HIV-  
1 in sample DNA, the bands are indistinct or not observed  
at all when the primers are not consistent with the  
10 subtype of the HIV-1 in sample DNA. The HIV-1 subtype is  
determined in this way.

[0039]

The method for determining the HIV-1 subtype of the  
present invention may include the steps of amplifying  
15 nucleic acid using as a target sequence a portion of a  
nucleotide sequence of the HIV-1 genome, wherein the  
nucleotide sequence is highly conserved among all subtypes,  
and ascertaining the presence or absence of HIV-1  
depending on whether or not the nucleic acid has been  
20 amplified. The step for ascertaining the presence or  
absence of HIV-1 may comprise amplifying the nucleic acid  
with a primer mixture for the first PCR (such as  
9AE/9B/12A/12B) using as a target sequence a portion of a  
nucleotide sequence of the HIV-1 genome, the nucleotide  
25 sequence being highly conserved among all subtypes, then

carrying out the second amplification reaction with a primer mixture for the second PCR (such as 10U/11LB/11LAE/11LC) using as a target sequence a nucleotide sequence within the above target sequence, and  
5 then ascertaining the presence or absence of HIV-1 depending on whether or not the nucleic acid has been amplified.

[0040]

The present invention also encompasses a kit for  
10 determining HIV-1 subtypes, comprising a primer pair in which a target sequence is a portion of a nucleotide sequence of the env gene of HIV-1 and at least one of the 5' terminal and 3' terminal nucleotide sequences is different depending on the subtype. Examples of primer  
15 pairs include primer pairs (inner primers) for the second PCR such as those above, and combinations of primer pairs for the first PCR (outer primers) and primer pairs for the second PCR. The kit of the present invention may also include dNTP mixtures, reaction buffers, DNA polymerase,  
20 universal subtype primer pairs for the first PCR (such as 9B/12B), and universal subtype primer pairs for the second PCR (such as 10U/11VB). To minimize the effects caused by inconsistencies between the primer and analyte HIV-1 DNA base pairs, the magnesium ion concentration in the  
25 reaction buffer should be increased from the usual

concentration of 1.5 mM to 4 mM.

[0041]

The components constituting the diagnostic kit may be packaged individually, assembled, or bundled in  
5 containers such as vials and tubes, and further the containers may be in supporting means divided for housing such components.

[0042]

[Examples]

10 The present invention is illustrated in detail with the following examples, but the scope of the present invention is not limited to these examples.

[Example 1]

Subjects and Method

15 1) Subtype-specific specimens to study method for determining subtype

Specimens were prepared by extracting DNA from the blood of 3 HIV-infected subjects determined to be subtype A, 8 HIV-infected subjects determined to be subtype B and  
20 3 HIV-infected subjects determined to be subtype E, by env gene sequencing and phylogenetic analysis.

2) Subjects for determining subtype

The HIV-1 subtype was determined using 8 HIV-infected patients who either visited or were hospitalized  
25 in hospitals in Tokyo.

[0043]

3) Preparation of DNA from blood of HIV-infected subjects  
10 mL peripheral blood was collected from the above  
HIV-infected subjects. Sodium citrate was used as an  
5 anticoagulant. Monocytes were separated from the  
peripheral blood by Ficoll-Paque (Pharmacia) density  
gradient centrifugation, and DNA was then prepared using a  
QIAamp Blood Kit (QIAGEN). The DNA was dissolved in pure  
water or buffer containing 1 mM EDTA, and was stored at  
10 -20°C until immediately before use. 0.5 µg DNA was used  
in PCR.

[0044]

4) Detection of subtypes A, B, and E by PCR

Figure 3 gives the nucleotide sequences of the  
15 primers used in PCR.

For subtype A-specific detection, nested PCR was  
carried out using 9AE and 12A as the primers for the first  
PCR, and 10 and 11QA as the primers for the second PCR.  
For subtype B-specific detection, nested PCR was carried  
20 out using 9B and 12B as the primers for the first PCR, and  
10 and 11BB as the primers for the second PCR. For  
subtype E-specific detection, nested PCR was carried out  
using 9AE and 12E as the primers for the first PCR, and 10  
and 11QE as the primers for the second PCR (Figure 3).

25 [0045]



PCR was carried out for 30 cycles, wherein one cycle consisted of 15 seconds at 94°C, 30 seconds at 56°C, and 1 minute at 72°C, with 100 µL reaction solution (10 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 1.0 µM primer, 2.5 units Taq polymerase) using 0.5 µg sample DNA prepared from HIV-infected subjects. Using 2 µL reaction solution obtained from the first PCR, the second PCR was carried out for 25 cycles under the same conditions with the exception of using 30 seconds at 60°C instead of 30 seconds at 56°C.

PCR products (subtypes A and E: 317 bp; subtype B: 342 bp) were detected by isolation through electrophoresis with 2% agarose gel, and by subsequent ethidium bromide staining.

[0046]

### Results

#### 1) Study of subtype determination by PCR of subtype-specific specimens

The following results were obtained for specimens whose subtypes had been determined by virus genome sequencing. Only subtype A specimens were positive, while subtype B and E specimens were all negative, in PCR using primers for the detection of subtype A. Only subtype B specimens were positive, while subtype A and E specimens were all negative, in PCR using primers for the detection

of subtype B. Only subtype E specimens were positive, while subtype A and B specimens were all negative, in PCR using primers for the detection of subtype E (Figure 4).

2) Determination of subtype of HIV-infected subjects by

5 PCR

Table 1 gives the results obtained in the determination of HIV-1 subtypes in 8 HIV-infected subjects who either visited or were hospitalized in hospitals in Tokyo.

10 [0047]

Table 1 The results obtained in the determination of unknown subtypes in 8 specimens

Case	primer pair for subtype A	primer pair for subtype B	primer pair for subtype E
P18	-	+	-
P19	-	+	-
P20	-	+	-
P21	-	-	+
P22	-	?	-
P23	-	-	+
P24	-	+	-
P25	-	+	-

In the table above, + denotes detection of HIV-1 specific DNA bands, - denotes non-detection thereof. The symbol "?" for case P22 denotes detection of shorter bands than expectation.

[0048]

Based on these results, Cases P18, P19, P20, P24, and P25 were diagnosed as being infected with subtype B,

and Cases P21 and P23 were diagnosed as being infected with subtype E. Although a DNA band was detected only with the use of a primer pair for subtype B in Case P22, it was shorter than expected, so determination was postponed. To verify that the above results were correct, the amplified DNA was sequenced and phylogenetically analyzed, showing that the results of the phylogenetic analysis were consistent with those in Table 1. Case P22, which was postponed, turned out to be subtype B.

10 [0049]

The results in 1) and 2) demonstrate that this method was able to correctly diagnose the subtype in 21 out of 22 cases. The determination was postponed in the remaining one case. That is, the present method has been shown to be a simple and reliable method for determining subtypes.

The method of the present invention allows the determination of HIV-1 subtype at a cost of about ¥2,000 per specimen. The time needed to determine the subtype in treating all 8 specimens at once was 2 hours for the isolation of DNA, 6 hours for PCR, and about 1 hour for electrophoresis.

[0050]

[Example 2]

25 Subjects and Method

1) Subtype-specific specimens to study method for determining subtype

Specimens were prepared by extracting DNA from the blood of 11 subjects, which included 2 patients infected with HIV-1 determined to be subtype C by env gene sequencing and phylogenetic analysis, in addition to the 3 patients infected with HIV-1 subtype A, 3 patients infected with HIV-1 subtype B, and 3 patients infected with HIV-1 subtype E used in Example 2.

10 [0051]

2) Subjects for determining subtype

The HIV-1 subtype was determined using 32 HIV-infected patients who either visited or were hospitalized in hospitals in Tokyo.

15 [0052]

3) Preparation of DNA from blood of HIV-infected subjects

10 mL peripheral blood was collected from the above HIV-infected patients. Sodium citrate was used as an anticoagulant. Monocytes were separated from the peripheral blood by Ficoll-Paque (Pharmacia) density gradient centrifugation, and DNA was then prepared using a QIAamp Blood Kit (QIAGEN). The DNA was dissolved in pure water or buffer containing 1 mM EDTA, and was stored at -20°C until immediately before use. 0.5 µg DNA was used in PCR.

25

[0053]

4) Detection of subtypes A, B, C, and E by PCR

Figure 5 gives the nucleotide sequences of the primers used in PCR.

5           A mixture of 9AE, 9B, 12A, and 12B was used as the primers for the first PCR. Nested PCR was carried out using the following primers for the second PCR: 10U and 11QA1 for subtype A-specific detection, 10U and 11VB for subtype B-specific detection, 10U and 11XC for subtype C-specific detection, and 10U and 11WE for subtype E-specific detection. Nested PCR was carried out using a mixture of 10U, 11LB, 11LAE and 11LC for amplifying HIV-1 DNA irrespective of subtype (Figure 5).

[0054]

15           PCR was carried out for 30 cycles, wherein one cycle consisted of 15 seconds at 94°C, 30 seconds at 56°C, and 1 minute at 72°C, with 100 µL reaction solution (10 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 1.0 µM primer, 2.5 units Taq polymerase) using 0.5 µg sample DNA prepared from HIV-infected subjects. Using 2 µL reaction solution obtained from the first PCR, the second PCR was carried out for 25 cycles under the same conditions with the exception of using 30 seconds at 60°C instead of 30 seconds at 56°C.

25           PCR products (subtype A: 322 bp; subtype B: 358 bp;

subtype C: 298 bp; subtype E: 361 bp)) were detected by isolation through electrophoresis with 2% agarose gel, and by subsequent ethidium bromide staining.

[0055]

5 Results

1) Study of subtype determination by PCR of subtype-specific specimens

The following results were obtained for specimens whose subtypes had been determined by virus genome sequencing. Only subtype A specimens were positive, while subtype B, C, and E specimens were all negative, in PCR using primers for the detection of subtype A. Only subtype B specimens were positive, while subtype A, C, and E specimens were all negative, in PCR using primers for the detection of subtype B. Only subtype C specimens were positive, while subtype A, B, and E specimens were all negative, in PCR using primers for the detection of subtype C. Only subtype E specimens were positive, while subtype A, B, and C specimens were all negative, in PCR using primers for the detection of subtype E (Figure 6). All specimens were positive in PCR using primers for amplifying HIV-1 DNA irrespective of subtype (Figure 6).

[0056]

2) Determination of subtype of HIV-infected subjects by  
25 PCR

The HIV-1 subtype was determined in 32 HIV-infected patients who either visited or were hospitalized in hospitals in Tokyo. Together with the 11 subtype-specific specimens, there were 3 cases of subtype A, 30 cases of  
5 subtype B, 2 cases of subtype C and 8 cases of subtype E.

To verify that the above results were correct, the amplified DNA of 21 out of the 43 HIV-infected patients whose subtype was determined by PCR was sequenced and phylogenetically analyzed, giving the results shown in  
10 Figure 7. There was complete agreement between the subtypes determined by phylogenetic analysis and the subtypes determined by PCR for HIV-1 of these cases.

[0057]

The method employed in Example 2 differs  
15 significantly from that in Example 1 in that universal primers were used in the first PCR, and subtype-specific primers were used in the second PCR. As a result, the number of PCR reactions could be reduced to 5/8. In view of the fact that the determination of the subtype had to  
20 be postponed in one case in Example 1, the method of Example 2 was able to provide more accurate and simpler diagnosis.

[0058]

3) Effect of subtype on drug resistance test by genotype  
25 Drug resistance test by genotype was analyzed based

on data for subtype B. To investigate whether or not the drug resistance of HIV-1 subtypes other than subtype B could be determined using data for subtype B, the amino acid sequences for HIV-1 protease before and after HAART treatment were determined in 4 patients infected with HIV-1 other than subtype B who were receiving HAART treatment. Figure 8 shows only the amino acids which are related to drug resistance to a protease inhibitor. In the case of Case 3 wherein the patients were infected with subtype E, after HAART treatment was started, amino acid No. 10 had mutated from L (leucine) to F (phenylalanine), and amino acid No. 20 had mutated from K (lysine) to T (threonine). This was recognized as an amino acid mutation indicative of drug resistance in subtype B. However, in all four patients, amino acid No. 36 was already I (isoleucine) before HAART treatment had started. From the data for subtype B, it was indicated that HIV-1 with I (isoleucine) as the amino acid No. 36 had drug resistance. However, it is difficult to conclude that HIV-1 would have acquired drug resistance before administration of the drug. Thus, it would be more logical to view this mutation as irrelevant to drug resistance in HIV-1 subtypes other than subtype B. It may thus be concluded that it is important to diagnose the subtype in advance in order to properly assess drug resistance by genotype.



[0059]

4) Relationship between subtype and sexual behavior

Figure 9 summarizes the relationship between subtype and sexual behavior in 22 HIV-infected patients who had been interviewed about their sexual preferences.

Heterosexuals are those attracted to the opposite sex, while MSM are male homosexuals. The numbers of subtype B and E were the same in heterosexuals, whereas the number of subtype B is much larger than that of subtype E in male homosexuals. This would seem to indicate that Southeast Asian-derived HIV has spread among heterosexuals but has not spread very much among male homosexuals.

[0060]

[Example 3]

15 Subjects and Method

1) Western blot-negative and PA-positive serum specimens

The specimens were 15 serum samples whose blood tests at hospitals in Tokyo showed to be HIV-1 negative by Western blotting and HIV-1 positive by PA.

20 2) Preparation of DNA from plasma RNA

RNA was prepared using an RNAeasy Kit (QIAGEN) from 200  $\mu$ L of the aforementioned serum specimen. The RNA was dissolved in pure water and stored at -20°C until immediately before use.

25 [0061]

3) Detection of HIV-1 by PCR

RNA corresponding to 20  $\mu$ L of serum was used as the material, and cDNA was synthesized by reacting for 30 minutes at 42°C using a mixture of primers 12A and 12B with 20  $\mu$ L reaction solution (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 1 mM dNTP, 5  $\mu$ M primer, and 100 units reverse transcriptase). The cDNA was used as material in nested PCR capable of amplifying the DNA of HIV-1 irrespective of subtype. A mixture of 9AE, 9B, 12A, and 12B was used as the primers for the first PCR. A mixture of 10U, 11LB, 11LAE and 11LC was used as the primers for second PCR.

[0062]

First PCR was carried out for 30 cycles, where one cycle consisted of 15 seconds at 94°C, 30 seconds at 56°C, and 1 minute at 72°C, with 100  $\mu$ L reaction solution (10 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 1.0  $\mu$ M primer, 2.5 units Taq polymerase). Using 2  $\mu$ L reaction solution obtained from the first PCR, the second PCR was carried out for 25 cycles under the same conditions with the exception of using 30 seconds at 60°C instead of 30 seconds at 56°C.

PCR products (subtype A: 322 bp; subtype B: 358 bp; subtype C: 298 bp; subtype E: 361 bp) were detected by isolation through electrophoresis with 2% agarose gel, and

by subsequent ethidium bromide staining.

[0063]

Results

No HIV-1 was detected by PCR in any of the 15 serum  
5 specimens which were determined to be HIV-1 negative by  
Western blotting and HIV-1 positive by PA (Figure 10). It  
was proven (Figure 6) that the PCR used here was designed  
to enable detection of all HIV-1 subtypes, and that the  
PCR was capable of detecting at least HIV-1 of subtypes A,  
10 B, C and E. It is highly possible that the inconsistency  
between the results by Western blotting and PA for the  
serum specimens tested here is not a problem of subtype,  
but a problem of false positive results by the PA method.

PCR, which is capable of detecting HIV-1  
15 irrespective of subtype, may therefore be more effective  
as a reliable diagnosis of HIV-1 infection.

[0064]

[Effect of the Invention]

The present invention provides a simple method for  
20 determining HIV-1 subtypes. The invention also provides  
an effective means for determining HIV-1 subtypes.

[0065]

[Sequence Listing]

<110> KEIO UNIVERSITY

<120> A METHOD FOR HIV-1 SUBTYPING

<130> P99-0608

5 <140>

<141>

<150> JP P11-167736

<151> 1999-06-15

10

<160> 34

<170> PatentIn Ver.2.0

15 <210> 1

<211> 20

<212> DNA

<213> Artificial Sequence

20 <220>

<223> Description of Artificial Sequence:synthetic DNA

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20

25

<210> 2

<211> 20

<212> DNA

5 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

10 <400>2

ctgtgcatta caatttctgg 20

<210> 3

<211> 20

15 <212> DNA

<213> Artificial Sequence

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5 <223> Description of Artificial Sequence:synthetic DNA

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10 <210> 5

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20

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20

25



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$\langle 220 \rangle$ 

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22

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[0066]

15 [Free Text of Sequence Listing]

Sequence ID No. 1 gives the nucleotide sequence for primer 11QA.

Sequence ID No. 2 gives the nucleotide sequence for primer 11BB.

20 Sequence ID No. 3 gives the nucleotide sequence for primer 11QE.

Sequence ID No. 4 gives the nucleotide sequence for primer 10.

25 Sequence ID No. 5 gives the nucleotide sequence for primer 12A.

Sequence ID No. 6 gives the nucleotide sequence for primer 12B.

Sequence ID No. 7 gives the nucleotide sequence for primer 12E.

5        Sequence ID No. 8 gives the nucleotide sequence for primer 9AE.

Sequence ID No. 9 gives the nucleotide sequence for primer 9B.

10       Sequence ID No. 10 gives the nucleotide sequence for primer 10C.

[0067]

Sequence ID No. 11 gives the nucleotide sequence for primer 10G.

15       Sequence ID No. 12 gives the nucleotide sequence for primer 10H.

Sequence ID No. 13 gives the nucleotide sequence for primer 11RC.

Sequence ID No. 14 gives the nucleotide sequence for primer 11RD.

20       Sequence ID No. 15 gives the nucleotide sequence for primer 11RF.

Sequence ID No. 16 gives the nucleotide sequence for primer 11SG.

25       Sequence ID No. 17 gives the nucleotide sequence for primer 11SH.

Sequence ID No. 18 gives the nucleotide sequence for primer 11LB.

Sequence ID No. 19 gives the nucleotide sequence for primer 11LE.

5        Sequence ID No. 20 gives the nucleotide sequence for primer 10U.

[0068]

Sequence ID No. 21 gives the nucleotide sequence for primer 10KC.

10       Sequence ID No. 22 gives the nucleotide sequence for primer 10UF.

Sequence ID No. 23 gives the nucleotide sequence for primer 10UG.

15       Sequence ID No. 24 gives the nucleotide sequence for primer 10UC.

Sequence ID No. 25 gives the nucleotide sequence for primer 11LAE.

Sequence ID No. 26 gives the nucleotide sequence for primer 11LC.

20       Sequence ID No. 27 gives the nucleotide sequence for primer 11QA1.

Sequence ID No. 28 gives the nucleotide sequence for primer 11VB.

25       Sequence ID No. 29 gives the nucleotide sequence for primer 11XC.

Sequence ID No. 30 gives the nucleotide sequence for primer 11WE.

[0069]

Sequence ID No. 31 gives the nucleotide sequence for  
5 primer 11TC.

Sequence ID No. 32 gives the nucleotide sequence for primer 11RC1.

Sequence ID No. 33 gives the nucleotide sequence for primer 11SE.

10 Sequence ID No. 34 gives the nucleotide sequence for primer 11BE.

[Brief Description of the Drawing]

[Figure 1]

Figure 1 illustrates nucleotide sequences of the 5' adjacent region (C2 region) of the V3 region for the env gene in various subtypes of HIV-1. Capital letters indicate that the nucleotides are entirely the same within a given subtype, and lower case letters indicate the presence of nucleotide variants that are different within a given subtype. A question mark "?" indicates that a consensus nucleotide was not determined because of too many variants. A dash "-" indicates a nucleotide identical to that in subtype A. A period "." indicates the absence of a nucleotide in the corresponding site.

25 [Figure 2]

Figure 2 illustrates nucleotide sequences of the 3' adjacent region (C3 region) of the V3 region for the env gene in various subtypes of HIV-1. Capital letters indicate that the nucleotides are entirely the same within a given subtype, and lower case letters indicate the presence of nucleotide variants that are different within a given subtype. A question mark "?" indicates that a consensus nucleotide was not determined because of too many variants. A dash "-" indicates a nucleotide identical to that in subtype A. A period "." indicates the absence of a nucleotide in the corresponding site.

[Figure 3]

Figure 3 illustrates the locations, combinations, and base sequences of primers used in nested PCR (different primer pairs used for the first and second PCR) for determining HIV-1 subtypes.

[Figure 4]

Figure 4 gives the results obtained when subtypes were detected by nested PCR using the primers illustrated in Figure 3 for specimens in which the subtypes had been determined by sequencing of the virus genome.

[Figure 5]

Figure 5 illustrates the locations, combinations, and base sequences of primers used in nested PCR (universal primers were used in the first PCR, whereas

different primer pairs were used for the second PCR) for determining HIV-1 subtypes. 9M indicates a mixture of primers 9AE and 9B; 11U indicates a mixture of primers 11LAE, 11B, and 11LC; and 12M indicates a mixture of  
5 primers 12A and 12B.

[Figure 6]

Figure 6 gives the results obtained when subtypes were detected by nested PCR using the primers illustrated in Figure 5.

10 [Figure 7]

Figure 7 gives the results of determination of subtypes by phylogenetic analysis of HIV-1 variants based on the base sequence of the V3 region of the env gene obtained through sequencing.

15 [Figure 8]

Figure 8 illustrates the amino acid sequences related to protease inhibitor resistance in patients with HIV-1 non-subtype B who receive HAART treatment.

[Figure 9]

20 Figure 9 is a table showing the relationship between various subtypes and the sexual behavior of HIV-1 patients.

[Figure 10]

Figure 10 gives the results obtained in RT-PCR using primer pairs allowing HIV-1 to be amplified irrespective  
25 of subtype in serum samples from patients diagnosed as



-72-

positive by particle adsorption ( $PA^+$ ) but negative by Western blotting ( $WB^-$ ). N1 and N2 are negative controls, while P1 and P2 are positive controls.



-73-

[Document Name] Drawing

[Figure 1]

Figure 1. The nucleotide sequences of C2 region in subtypes of HIV-1

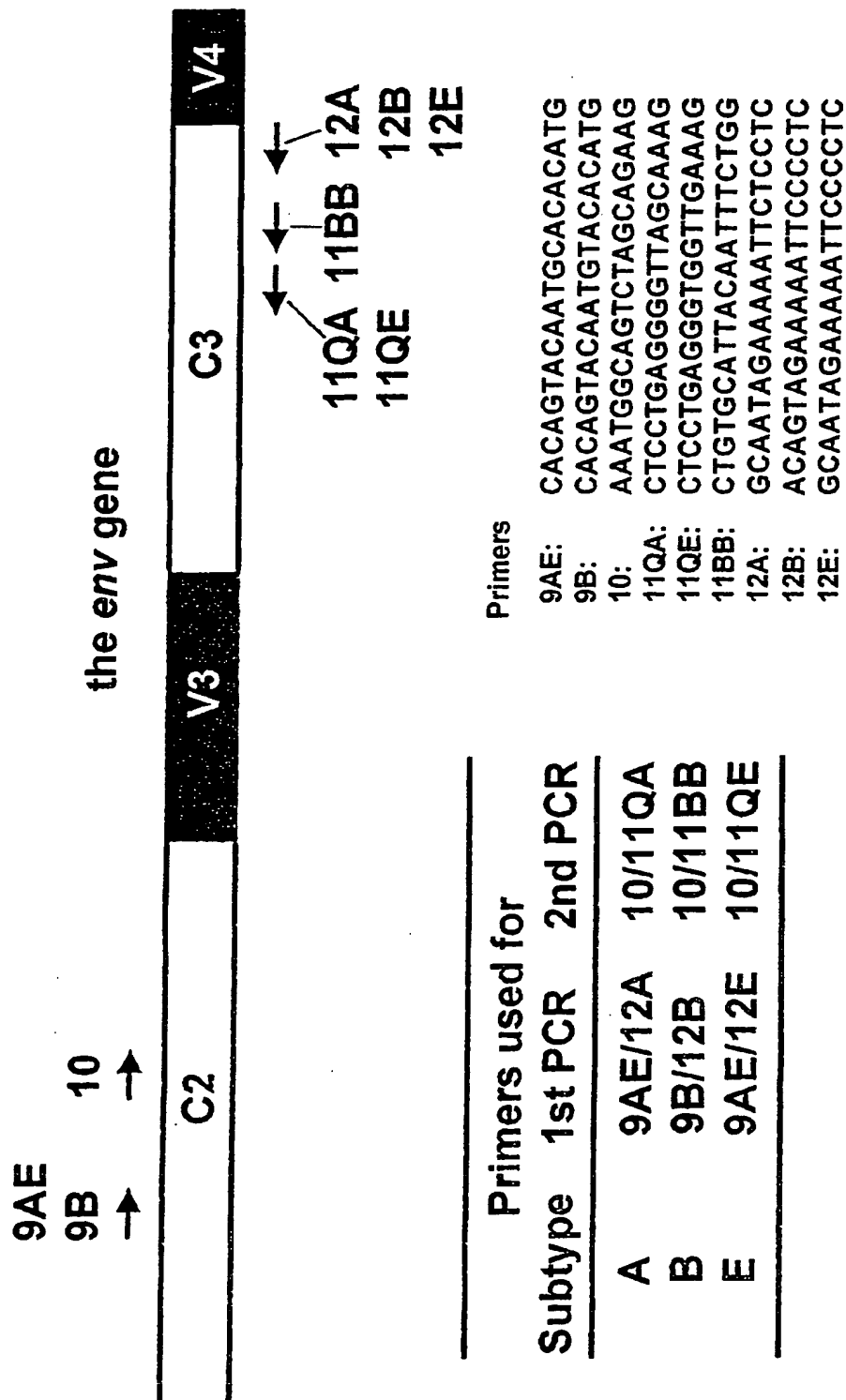
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SUBTYPE B	-----c-----t-----c-----
SUBTYPE C	-----A-----a-C-----c-t-----c-----T-----
SUBTYPE D	-----g-----a-----
SUBTYPE E	-----T-----T-----aG-----a-----T-----t-----
SUBTYPE F	-----A-----T-----GG-----T-----
SUBTYPE G	-----gt-----A-----ga?T-----c-----
SUBTYPE H	-----GT-----A-----GAGT-----T-----
SUBTYPE A	caTTATTGtgCcCCaGCTGGtTtTGCGaTtCTAAagTGtAa?gataaggagTTcaatGGA
SUBTYPE B	-----g-----t-----a-----
SUBTYPE C	-----t-----a-----ta-----aca-----g-----
SUBTYPE D	-----a-----a-----a-----A-----g-----
SUBTYPE E	-----a-t-----a-----t-----T-----a-t-----g-----
SUBTYPE F	-----T-----A-----T-----aA-----G-----
SUBTYPE G	-----T-----t-----gg-----a?-----
SUBTYPE H	-----T-----G-----A-----GG-----A-----G-----
SUBTYPE A	acAGGgccatGcaagAATGTcAGcaCaGTaCAATGcACacATGGaATcAagCCAGtagTa
SUBTYPE B	-----a-----t-ca-----t-----t-g-----
SUBTYPE C	-----a-----c-t-----t-----t-----g-----
SUBTYPE D	-----?-----a-----t-----g-t-g-----g-----
SUBTYPE E	-----t-A-----T-----T-----T-----G-----
SUBTYPE F	-----g-----T-----T-----T-----A-----g-----
SUBTYPE G	-----A-----T-a-----T-----T-----T-----g-----
SUBTYPE H	-----G-AA-----T-A-----T-----T-----T-----G-----
SUBTYPE A	tCAACTCAaCTgcTGtTaAATGGcAGtcTAGCagaAgaa???gaggTAatgaTtagaTCT
SUBTYPE B	-----a-----t-c-----...-g-a-----
SUBTYPE C	-----?-----g-----...-a-----A-----
SUBTYPE D	-----t-----A-----A-----A-----C-----
SUBTYPE E	-----T-T-----C-----...-ta-----A-----c-----
SUBTYPE F	-----t-a-----c-g-----t-----...-aA-----a-----
SUBTYPE G	-----T-A-----C-GTCAAAATG-CAGTTT-C-----?a-----a-----
SUBTYPE A	gAaantaTcacAaAcAATgccaaaAccaTAaTaGTacAgcTtg??aagcctGTAA?aATt
SUBTYPE B	-----t-----gg-----t-----gaa-g-at-----ga-----
SUBTYPE C	-----c-g-----t-----a-----t-----aAtg-at-----ga-----
SUBTYPE D	-----c-----t-----?-----AAtG-t-----?c-----
SUBTYPE E	-----C-----G-C-----AAT-At-----Ga-----C-----
SUBTYPE F	c-----t-g-t-----A-----?-----AATg-At-----ca-----
SUBTYPE G	-----c?-----g-----gt-----g-----AAt-a?-a-ga-----
SUBTYPE H	-----c-----g-----a-----gt-----AAt-a?-g-----
SUBTYPE A	aatTGT
SUBTYPE B	-----
SUBTYPE C	gtg---
SUBTYPE D	-----
SUBTYPE E	-----
SUBTYPE F	-----
SUBTYPE G	---?---
SUBTYPE H	---?---

[Figure 2]

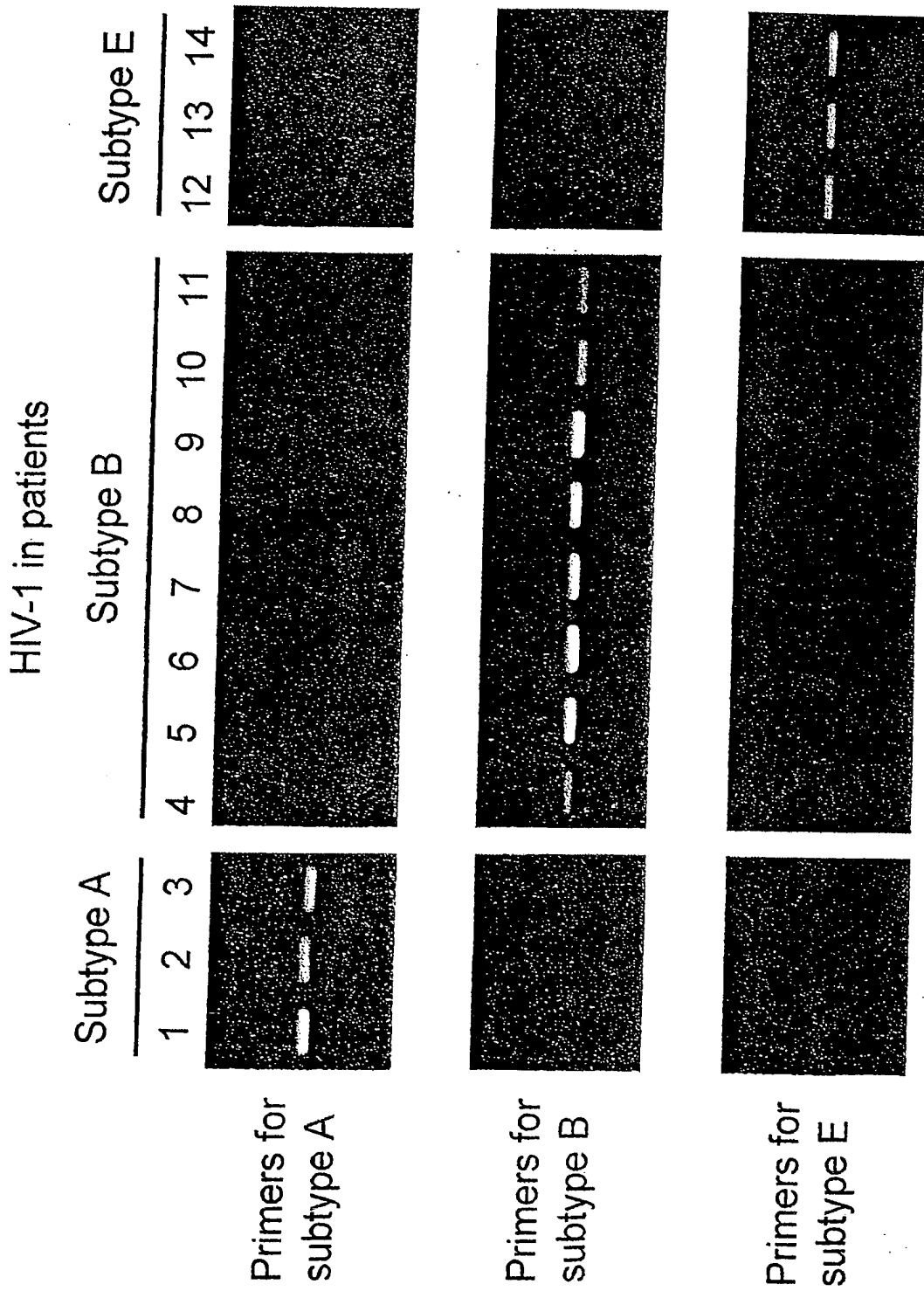
Figure 2. The nucleotide sequences of C3 region in subtypes of HIV-1

SUBTYPE A	TGTaatgTcAgtaga?cagaaTGGaAataaaacttTacaa?aggtagcta?acAatTAaga
SUBTYPE B	----ca-t----g-a-----c-----a-c-a--t---A-----
SUBTYPE C	----CA-T----a-ga?a-----?-----a-----ag-a-a-----gc-
SUBTYPE D	----a-T----a-ga?a-----c-----a-a-----g-
SUBTYPE E	---G-gA-T-A-g-A-a-----g-g-----a-c-----a-ga-a-----a-
SUBTYPE F	----c-t--g-a-C-----?--?--g-a-----a?ggc-a-g---ag
SUBTYPE G	-----t-----a?-a-t-----?g-g-tG-----ga-t--?a??gc?-C--a-
SUBTYPE H	-----T-----g?-a-?-----g-g-tg---?--?a-----?--?c-----?a-
SUBTYPE A	aaa.....tacTtt?????????aacaaaaca...??????ataatcTTtgctaac...
SUBTYPE B	g--?????c-a---...g-g-----t-----g-----aa-c-a???
SUBTYPE C	g--.....c---ccct-----T-----aa-----acca...
SUBTYPE D	g-c?.....cTtc-----...aca-----t---aaacCa...
SUBTYPE E	g-g.....C-----a-t--T-G-----caaCCA???
SUBTYPE F	tct.....c-t--c-----tg-----aa---aactcA...
SUBTYPE G	g--.....at-----?c???-----c---aaCtCA...
SUBTYPE H	---.....?-----a---t---??.....c---aaacca???
SUBTYPE A	?cctcaGGaGGGGAt?TaGAAaTtacAAcacAtAgttTTAaTTGTggAgGagaattttTtc
SUBTYPE B	t-----cCc-----gt--tg--c-----g-----
SUBTYPE C	t-----cc-----c-----a-----
SUBTYPE D	t-----ccc-----c-----g-----
SUBTYPE E	c-----a--C-----tg--ca-----A--g-----
SUBTYPE F	t-----CC-----tg-----a-----
SUBTYPE G	t-tg-----cC-----a-----
SUBTYPE H	t-----Cc-----?-----a-----
SUBTYPE A	TAtTGc
SUBTYPE B	--c--t
SUBTYPE C	-----
SUBTYPE D	--C---
SUBTYPE E	-----
SUBTYPE F	--C---
SUBTYPE G	-----t
SUBTYPE H	-----t

[Figure 3]



[Figure 4]

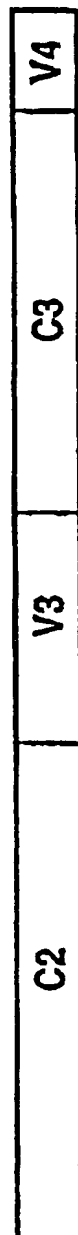


# Location of primers in HIV-1 subtype-specific nested PCR

[Figure 5]

the env gene

9M 10U  
→ →



← ← ← ←  
11XC 11QA 12M

←  
11VB

← ←  
11M 11WE

Primers used for

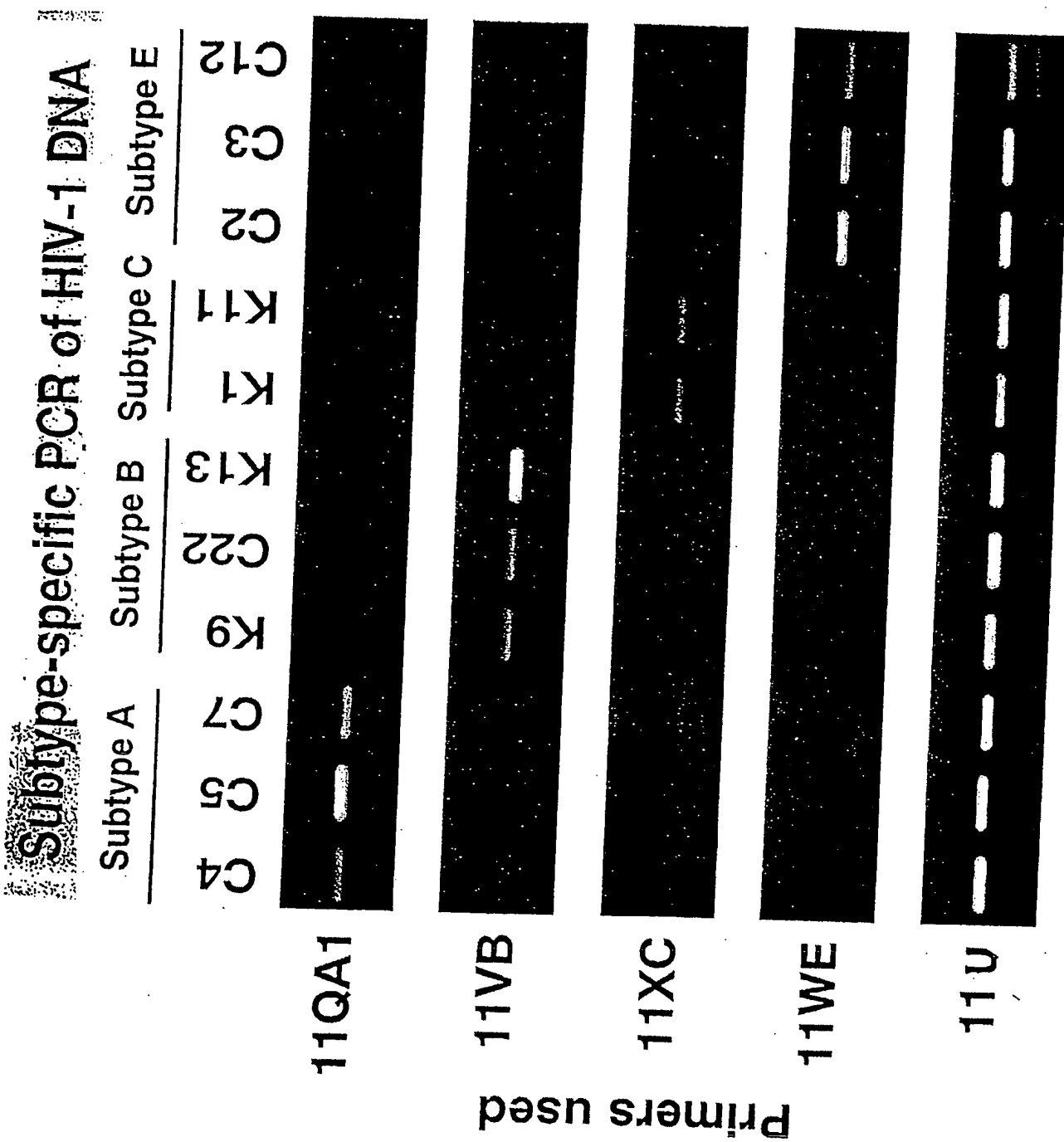
Subtype 1st PCR 2nd PCR

A	9M/12M	10U/11QA1
B	9M/12M	10U/11VB
C	9M/12M	10U/11XC
E	9M/12M	10U/11WE
any	9M/12M	10U/11M

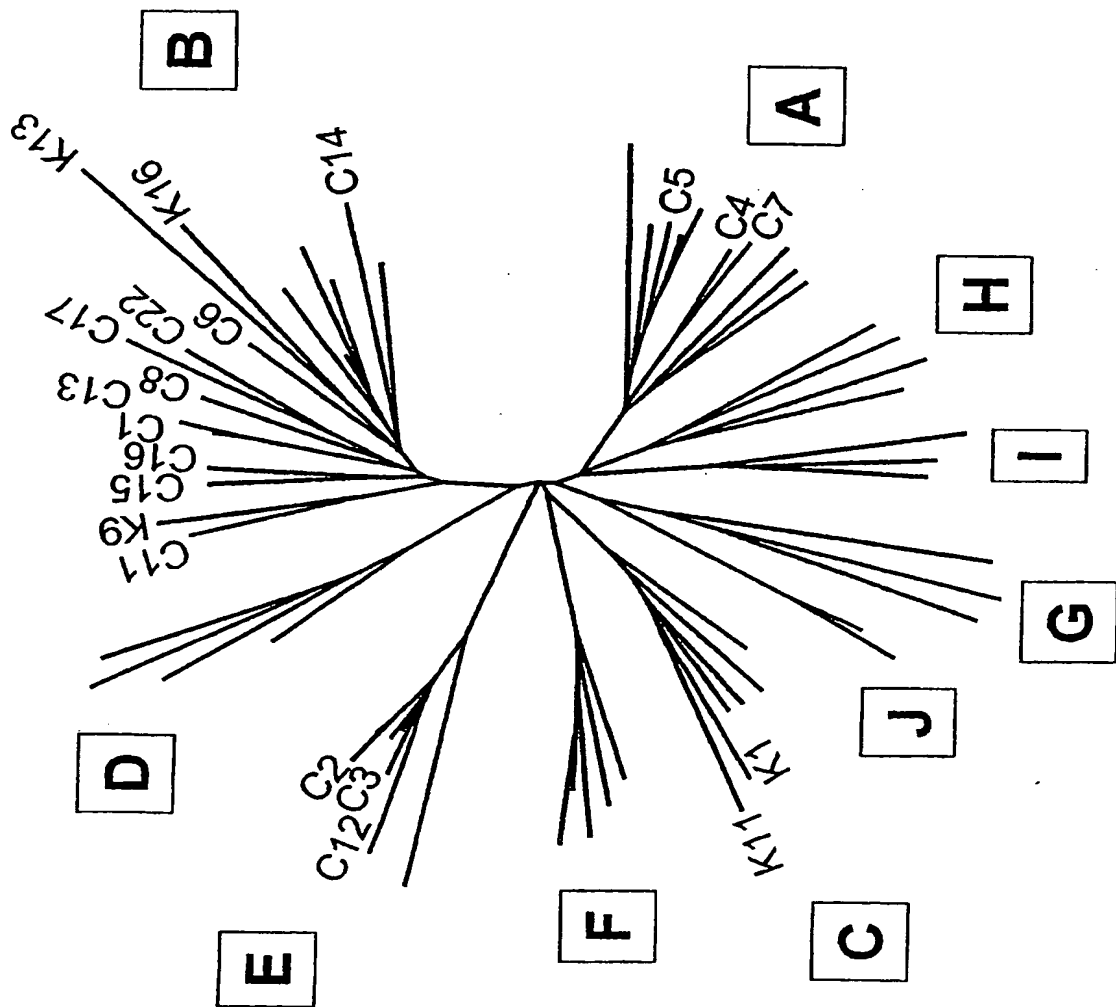
Primers

9AS: CACGTCACATGCCACATG  
9B: CACGTCACATGCCACATG  
100: CTGTTAATCCAGTCAGC  
11QA1: CACCTGAGGAGTTCGAAAG  
11VB: CACAAITAAACGTGCAATAC  
11XC: TTGTTTATAGGAGGTTC  
11WE: CTTTACAAITAAATGTCATTC  
11M: AATTTACATCCCTCTG  
11B: AATTTCCCTCCCTCTG  
11L: AATTTCCCTCCCTCTG  
12A: GCAATAGAAATTTCTCTC  
12B: ACAGTACAAATTTCTCTC

[Figure 6]



[Figure 7]





# Amino acid sequence in PR of non-subtype B HIV-1 in patients receiving HAART

[Figure 8]

-80-

Patient	Subtype	Date	Amino acid at positions relevant to PRT resistance													
			10	20	30	36	46	48	50	63	82	84	90			
C3	E	8/11/97	L	K	D	I	M	G	I	L	V	I	L			
		6/17/99	F	T	D	I	M	G	I	L	V	I	L			
C4	A	6/9/97	L	I	D	I	M	G	I	N	V	I	L			
		3/11/98	L	I	D	I	M	G	I	N	V	I	L			
C5	A	6/23/97	L	I	D	I	M	G	I	P	V	I	L			
		1/11/99	L	I	D	I	M	G	I	P	V	I	L			
C7	A	7/29/97	L	I	D	I	M	G	I	N	V	I	L			
		9/16/99	L	I	D	I	M	G	I	N	V	I	L			

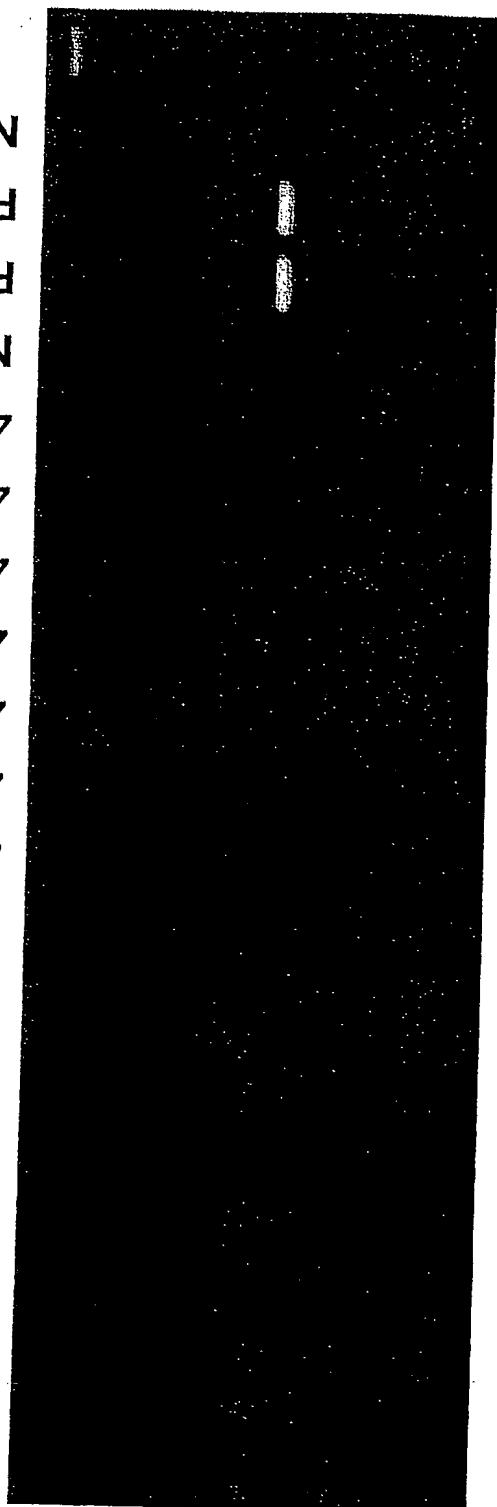
# Sexual behavior and HIV-1 subtype

[Figure 9]

	Heterosexual	MSM	Total
Subtype A	2	1	3
Subtype B	6	17	23
Subtype C	2	0	2
Subtype E	6	1	7
Total	14	19	33

**RT-PCR of RNA from PA<sup>+</sup> but WB<sup>-</sup> plasma with  
universal primers**

4320  
4361  
4269  
4081  
4378  
4589  
4317  
4494  
4727  
4309  
4017  
4441  
4488  
4480  
4091  
N1  
P1  
P2  
N2



[Document Name] Abstract

[Abstract]

[Object] A method for determining HIV-1 subtypes, which comprises the steps of amplifying nucleic acid using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1 wherein at least one of the 5' terminal and 3' terminal nucleotide sequences is different depending on the HIV-1 subtype, and detecting the subtype depending on whether or not the nucleic acid has been amplified. A kit for determining HIV-1 subtypes, comprising a primer pair in which a target sequence is a portion of a nucleotide sequence of the env gene of HIV-1 wherein at least one of the 5' terminal and 3' terminal nucleotide sequences is different depending on the subtype.

[Method for Achieving the Object] The present invention provides a simple method for determining HIV-1 subtypes. The present invention also provides an effective method for determining HIV-1 subtypes.

[Selected Figure] None